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Caught in the evolutionary act: Precise *cis*-regulatory basis of difference in the organization of gene networks of sea stars and sea urchins

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Abstract

The regulatory control of *otxβ1/2* in the sea urchin *Strongylocentrotus purpuratus* and the sea star *Asterina miniata* provides an exceptional opportunity to determine the genomic basis of evolutionary change in gene regulatory network (GRN) architectures. Network perturbation analyses in both taxa show that Otx regulates the transcription factors *gatae* and *krox/blimp1* and both of these transcription factors also feed back and regulate *otx*. The *otx* gene also autoregulates. This three way interaction is an example of a GRN kernel. It has been conserved for 500 million years since these two taxa last shared a common ancestor. Amid this high level of conservation we show here one significant regulatory change. Tbrain is required for correct *otxβ1/2* expression in the sea star but not in the sea urchin. In sea urchin, *tbrain* is not co-expressed with *otxβ1/2* and instead has an essential role in specification of the embryonic skeleton. *Tbrain* in these echinoderms is thus a perfect example of an orthologous gene co-opted for entirely different developmental processes. We isolate and test the sea star *otxβ1/2 cis*-regulatory module and demonstrate functional binding sites for each of the predicted inputs, including Tbrain. We compare it to the logic processing operating in the sea urchin *otxβ1/2 cis*-regulatory module and present an evolutionary scenario of the change in Tbrain dependence. Finally, inter-specific gene transfer experiments confirm this scenario and demonstrate evolution occurring at the level of sequence changes to the *cis*-regulatory module.

Keywords: *Cis*-regulatory evolution; Sea urchin; *Asterina miniata*; Gene regulatory network

Introduction

Logic and much indirect evidence identify *cis*-regulatory sequence change as the predominant mechanism underlying the evolutionary divergence of development (Britten and Davidson, 1971; Belting et al., 1998; Sucena et al., 2003; Shapiro et al., 2004; Davidson, 2006; Marcellini and Simpson, 2006). But actual evidence of addition or subtraction of transcription factor target sites during evolution has remained elusive. This is because of the enormity of data needed to convincingly demonstrate evolutionary loss and gain of regulation at the *cis*-level. *Cis*-regulatory modules from two appropriately chosen organisms must be isolated and functionally characterized. The surrounding gene regulatory network (GRN) architecture from both taxa must be well enough known that predictions of loss and gain in transcription factor regulation can be reliably made. Ideally, the GRN architecture comparison should predict a specific regulatory change among a background of conserved regulatory connections. Finally, the phylogenetic relationship of the chosen taxa must be well resolved.

We report here a comparative analysis of the *cis*-regulatory control of the *otx* gene in sea stars and sea urchins in which all of these criteria have been met. We provide functional analysis of the *cis*-module regulating *otxβ1/2* expression in the sea star and compare its structure and logic processing to that published for the equivalent regulatory module in the sea urchin.

Alternatively processed *otx* transcripts exist in the two divergent echinoderms, the sea star *Asterina miniata* and sea urchin *Strongylocentrotus purpuratus*, and are widely expressed during early development (Gan et al., 1995; Li et al., 1997; Yuh et al., 2002; Hinman et al., 2003b). The *otxβ1/2* transcript in both animals is expressed in the endomesoderm and ectoderm

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from early blastula stage. *A. miniata otxβ3* (*Amotxβ3*) is present maternally and expressed solely in the ectoderm of the blastula and gastrula. *Amotxα* expression is confined to the endomesoderm, and like *Amotxβ1/2*, is first detected in the blastula. The orthologous *S. purpuratus otx* (*Spotx*) transcripts are very similarly spatially restricted in the sea urchin, although there are differences in their temporal onset. *Spotxα* is present maternally, while *Spotxβ3* and *Spotxβ1/2* are zygotically activated. Thus in *A. miniata*, in contrast to *S. purpuratus*, there is no maternally expressed *otx* found later in the endomesoderm.

The GRN surrounding the *otx* gene has also been well documented. In both these echinoderms Otx regulates the transcription factor genes *gatae* and *krox/blimp1* and both of these factors also feed back and regulate *otxβ1/2* (Davidson et al., 2002a;b; Hinman et al., 2003a). Otx is also positively autoregulatory. The interaction of these genes to form a three way feedback loop is an example of a developmental GRN kernel and has been conserved in function throughout the 500 million years since these two echinoderms last shared a common ancestor (Hinman et al., 2003a; Davidson and Erwin, 2006). The consequence of this regulatory feedback kernel is stabilization of the regulatory state of the endomesoderm, and in particular the continued expression of *gatae* and *otx*, which are required for the expression of many other genes downstream in the endodermal GRN (Davidson et al., 2002a,b; Hinman et al., 2003a; Davidson, 2006). Yuh et al. (2004) have confirmed that, in the sea urchin, each of the regulatory connections predicted by the GRN (i.e. the regulation of *otxβ1/2* by Krox/Blimp1, Gatae and Otx itself) is the consequence of direct binding of these proteins to the *cis*-regulatory element. It might be predicted that the mechanism underlying the conservation of this GRN kernel must be the maintained requirement for the binding of the orthologous regulatory proteins to the *cis*-regulatory DNA of the *A. miniata otxβ1/2* target gene.

Amid this high conservation of regulatory inputs we also show here one evolutionary difference in the regulation of *otxβ1/2*. Expression of the sea star, but not sea urchin, *otxβ1/2* transcript requires correct *tbrain* function. *Tbrain* in sea stars is spatially co-expressed with *otxβ1/2*. In contrast, *Tbrain* expression in the sea urchin occurs only in the primary mesenchyme cells that ingress into the blastocoel (Croce et al., 2001). It has an obligatory role in specification of these cells into an embryonic skeletogenic phenotype (see http://sugp.caltech.edu/endomes/). In sea urchins, *tbrain* is not co-expressed with *otxβ1/2* and consequently has no direct role in its regulation. Tbrain in these echinoderms thus provides a perfect example of an orthologous gene co-opted for entirely different developmental processes. *Tbrain* orthologues are broadly expressed throughout endomesodermal tissues in sea cucumbers, hemichordates, amphioxus and many vertebrates (Maruyama, 2000; Tagawa et al., 2000; Horton and Gibson-Brown, 2002; Satoh et al., 2002). Therefore lack of *tbrain* expression within the endomesoderm most likely represents an evolutionary loss specific to the echinoid lineage. While we do not know whether Tbrain is required to regulate *otx* in all of these other deuterostomes, the expression patterns suggest that Tbrain regulation of *otx* is also ancestral, while loss of regulation is derived.

Materials and methods

Cloning of the A. miniata otx BAC recombinant

An *A. miniata* genomic library was constructed in the BACe3.6 vector (Frengen et al., 1999) with an average insert size of approximately 180 kb, following the protocol of Cameron et al. (2000) and probed with an 836 bp of fragment the *A. miniata otx* cDNA using standard hybridization protocols. One of the clones selected (reference # 116H21) had an insert size of approximately 165 kb and was sequenced by the Joint Genome Institute and annotated using the SUGAR software package (Brown et al., 2002). An *otx* GFP BAC recombinant was made following the protocol of Yu et al. (2000) in which the GFP coding sequence replaced the first coding exon of *otxβ1/2*.

A. miniata expression vectors

Two new expression vectors based upon the pGL3 GFP reporter construct adapted for use in the sea urchin (Arnone et al., 1997; Cameron et al., 2004) were developed for use in *A. miniata*. We inserted an 87 bp region corresponding to the sea urchin hatching enzyme gene's basal promoter (Wei et al., 1995) upstream of GFP. This vector was named HbGFP. A second expression vector, OpGFP, was constructed by replacing the hatching enzyme basal promoter in HbGFP with the sequence corresponding to the 476 bp directly upstream of the *Amotxβ1/2* transcript start, which included the putative endogenous basal promoter.

Identification of cis-regulatory module regulating otxβ1/2 expression

The web based Cluster Buster software (Frith et al., 2003) was used to look for clusters of consensus binding sites for Gata 5′-(A/C/T)GATA(A/G) (Lowry and Atchley, 2000), Otx 5′-TAATC(T/C) (Driever and Nusslein-Volhard, 1989) and Krox/Blimp1 5′-G(A/G)AA(C/G)(G/T)GAAA or 5′-G(A/G)AA(C/G)(G/T)AAA (Gupta et al., 2001; Marecki and Fenton, 2002). 100 kb (the maximum allowed in the analysis) of sequence surrounding the *otx* gene (starting 6.3 kb upstream of the first exon and approximately 62.3 kb downstream of the last known exon) was used for the analysis. The following parameters were used: Gap Parameter—100, Residue Abundance Range—400, Cluster Score Threshold—3, Motif Score Threshold—5, Pseudocount—0, and Filters—none.

These same parameters identified the known regulatory module in the sea urchin *otx* BAC recombinant sequence.

The presence of consensus binding sites for Tbrain (A/G)GGTG(T/C)GA defined by Conlon et al. (2001) for *Xenopus* was also sought by visual inspection of the cluster sequences. To ensure that we identified all potential Tbrain sites we also looked for the less specific site (A/G/T)(A/G/T)GTG(A/C/T)NA which binds Tbox orthologues including Tbrain from a variety of species (Heicklen-Klein and Evans, 2004).

Making and microinjection of reporter constructs

The seven highest scoring clusters (as defined by Cluster Buster) with binding sites for all three factors (*viz* Otx, Krox/Blimp1 and Gata) were PCR amplified and ligated into the HbGFP expression vector. These constructs are named OtxA- through OtxG-HbGFP.

Cluster OtxG, which was found to correctly drive reporter expression, was also ligated into EpGFPII (Cameron et al., 2004) and OpGFP (see above).

The expression constructs were linearized with *Kpn*1 or *Sac*1 and approximately 2000 copies were injected into fertilized zygotes along with an equivalent molar amount of *Kpn*1 digested *A. miniata* genomic DNA in a 200 mM KCl solution, following the microinjection protocol of Hinman et al. (2003b). An *otx* BAC GFP recombinant was made by replacing GFP coding sequence with the first coding exon of *otxβ1/2*. This construct was linearized with Asc1 and 100–200 copies in 200 mM KCl were injected into one cell zygotes.

Perturbation strategy

One of the seven constructs, OtxG-HbGFP, drove correct reporter expression. Core motifs of predicted binding sites were mutated (Supplemental Fig. 1). Thus Gata sites were changed from (A/C/T)**GATA**(A/G) to (A/C/T)

ACCG(A/G), Otx sites were changed from **TAAT**C(T/C) to **GCCG**C(T/C), Krox/Blimp1 sites were changed from (A/G)**AA**(C/G)(G/T)(G)A**AA** to (A/G)**CC**(C/G)(G/T)(G)**GGG** and Tbrain sites were changed from (A/G/T)(A/G/T)**GTG**(A/C/T)N**A** to (A/G/T)(A/G/T)**ACA**(A/C/T)N**C**. Mutagenesis was achieved using fusion PCR. Mutations of combinations of predicted sites were achieved by subsequent rounds of fusion PCR using the previously mutated templates.

Trans-perturbation was achieved by blocking the translation of the *tbrain* transcript using a morpholino substituted antisense oligonucleotide (MASO) designed to bind across the start of translation of AmTbrain (AAGCATACTC-GATACAGATCCAAAC). 400–600 μM of this MASO was injected into zygotes. AmTbrain MASO was also added to the injection solutions of either OtxG-HbGFP or the Otx BAC GFP recombinant and effects on GFP reporter expression were assayed as described below.

Analysis of GFP expression and quantitative real time PCR (QPCR)

Following injection of expression constructs, embryos were assayed visually for GFP using fluorescence microscopy or using whole mount in situ hybridization with DIG-labeled antisense GFP riboprobe following the protocol of Minokawa et al. (2005).

GFP transcript abundance was measured using quantitative real time PCR (QPCR) following protocols established for use in the sea urchin (Revilla-i-Domingo et al., 2004), with the following exceptions: RNA was extracted from 30 to 100 embryos using Sigma's GenElute™ Mammalian Total RNA Miniprep Kit and reverse transcription was achieved using iScript (Bio-Rad). Transcript abundance of targeted genes was normalized to sea star *ubiquitin* mRNA.

Tbrain quantitative developmental time series was achieved following the protocol of Hinman et al. (2003b).

Cloning and modification of the sea urchin cis element regulating Spotxβ1/2

The sea urchin regulatory modules SpOtx15 and 16 (Yuh et al., 2002, 2004), were cloned into pGL3 so that module SpOtx15 lay upstream of eGFP and module Otx16 downstream of the SV40 polyadenylation coding sequence. This module was modified by inserting either a 165 bp region of the sea star OtxG element (containing one binding site for Gata, one for Otx and three for Tbrain; see Supplemental Fig. 2) into the SpOtx15 or this same OtxG insert in which the three Tbrain sites were mutated as described above. This insertion was achieved using fusion PCR. Briefly, three PCR products were obtained, one corresponding to the region of SpOtx15, 5′ to the desired insertion site, one corresponding to the region of SpOtx15 3′ to insertion site and one of the OtxG or OtxG with mutated Tbrain sites region targeted for insertion. The two products corresponding to SpOtx15 were engineered with 12 bp of sequence corresponding to either the 5′ or 3′ sequence of the OtxG insert as appropriate, while the OtxG insert was engineered with 12 bp tails on either end corresponding to the SpOtx15 sequence at the point of insertion. All three products were used as template in a further PCR using primers flanking SpOtx15. This insert was then cloned into pGL3 already containing the downstream module16.

Results

Tbrain regulates otxβ1/2 in the sea star

In the sea star, *tbrain* is expressed strongly throughout the endomesoderm in blastula and gastrula stages (Figs. 1A, B). Expression is also detected throughout the ectoderm, although transcripts are less abundant than in the endomesoderm. *Tbrain* transcripts start accumulating between 8 and 12 h (Fig. 1C), compared to *otxβ1/2* which is first expressed several hours later at 15 to 18 h (Hinman et al., 2003b). QPCR analyses demonstrate that *otx*α and *otxβ1/2* transcripts are reduced more than three fold in Tbrain MASO injected versus control MASO injected embryos (Fig. 1D). Conversely, *otxβ3*, which is not expressed in the endomesoderm, does not depend on correct *tbrain* function.

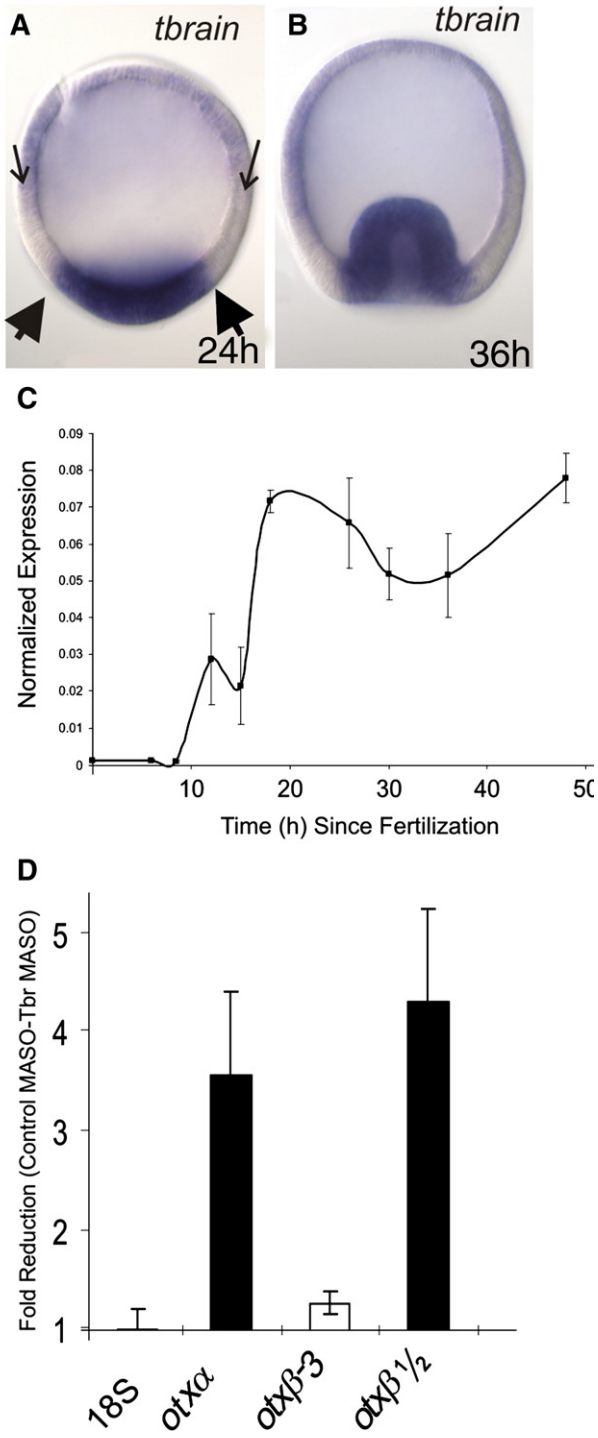

Fig. 1. Tbrain in *A. miniata*. (A, B) Expression of Tbrain in *A. miniata* blastula (A) and early gastrula (B) embryos shown using whole mount in situ hybridization. Strong expression is found throughout the endomesoderm (arrowheads) and weaker expression throughout the animal ectoderm (arrows). (C) Developmental time series of *tbrain* expression normalized to *ubiquitin*. (D) Fold reduction in *otx* transcript abundance in Tbrain deficient 24 h old embryos. Bars are ±1 standard deviation.

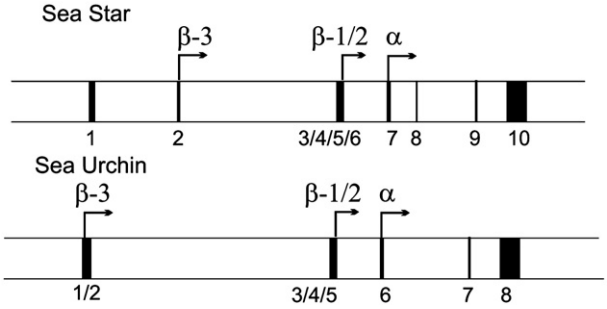

Fig. 2. Schematic of the *A. miniata* and *S. purpuratus otx* genes. Exons (black boxes) are numbered and arrows indicate the start of translation of each of the three protein forms. Multiple exons may be displayed as a single line (e.g. exons 3, 4, 5).

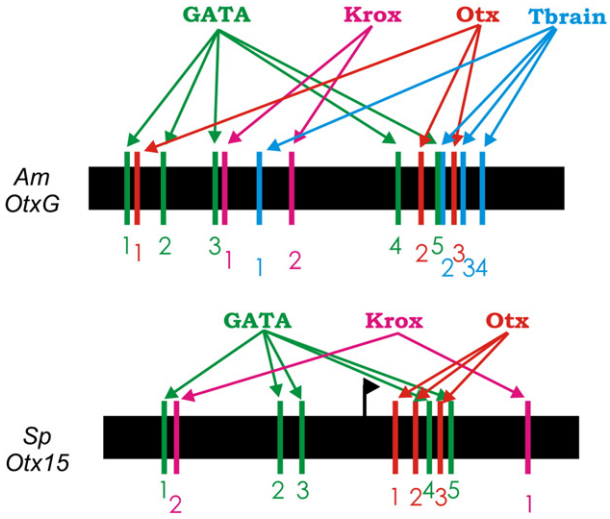

Fig. 3. A schematic of the binding site distribution in *cis*-regulatory modules regulating embryonic expression of *otxβ1/2* in the sea star (AmOtxG) or the sea urchin (SpOtx15). Each module contains five consensus binding sites for Gata (green), Otx (red) and Krox/Blimp1 (pink). OtxG also contains four Tbrain sites (blue). Black arrow shows start of transcription in SpOtx15.

The genomic structure of the A. miniata otx gene

A BAC recombinant containing the *A. miniata otx* gene (reference # 116H21) was isolated and sequenced. Analysis of this sequence revealed that the *A. miniata otx* gene consists of ten exons. The first two are found exclusively in Otxβ3, exons 3,4, 5 and 6 are used only by Otxβ1/2, and exon 7 only by Otxα (Fig. 2). The translation start for Otxβ1/2 is found in exon 6, with exons 3, 4 and 5 being alternatively used to produce three different 5′UTR sequences. For the sake of simplicity each of these three transcripts is named *otxβ1/2* as they differ only in slight variations in 5′UTR. The three C terminus coding exons (8, 9, 10) are shared by all transcripts, with the homeobox located on exons 9 and 10. This is a similar organization and ordering of exon usage as found for the *S. purpuratus otx* gene (Yuh et al., 2002) except that there are two additional exons in *A. miniata*. One of these additional exons, either 3, 4 or 5 (there is not sufficient sequence identity to determine direct orthology), is used to produce an additional alternative transcript coding Otxβ1/2; there are only two alternative transcripts coding Otxβ1/2 in sea urchin. The other extra exon (exon 8) is only 9 nucleotides long.

Identification of OtxG, a cis-regulatory module regulating otxβ1/2 expression

The first objective was to identify the *cis*-regulatory element responsible for control of *Amotxβ1/2* expression during blastula and early gastrula stages. We have shown previously (Hinman et al., 2003a) that *otxβ1/2* is regulated by Gatae, Blimp1/Krox and itself, so we searched for clusters of the predicted binding sites for these proteins. The software used to identify potential modules, Cluster Buster (Frith et al., 2003), allows the selection of several parameters affecting the size of the module and distribution of binding sites within it. We used 100 kb of sequence surrounding the sea urchin *otx* gene in a test analysis and systematically modified these parameters until the known regulatory module, SpOtx15, was identified. These same parameters were used to search for clusters in 100 kb of sequence surrounding the sea star *otx* gene. Seven clusters were found that contained binding sites for Gata, Blimp1/Krox and Otx, ranging in size from 74 bp to 603 bp, and located from approximately 5 kb upstream of the first exon to 19 kb downstream from the last. Each of these clusters was cloned into an expression construct (HbGFP; containing the basal promoter for hatching enzyme and a GFP reporter) and tested for its ability to drive correct reporter gene expression. Only one construct, OtxG-HbGFP, drove any expression. Module OtxG, located approximately 11 kb downstream of the last *otx* exon, was 591 bp long as predicted from Cluster Buster analysis but a slightly larger region of 854 bp was cloned into HbGFP (Supplemental Fig. 1A). This cluster contained five predicted binding sites for Gata, three for Otx, two for Blimp1/Krox and also four for Tbrain/Tbox including one instance of the very

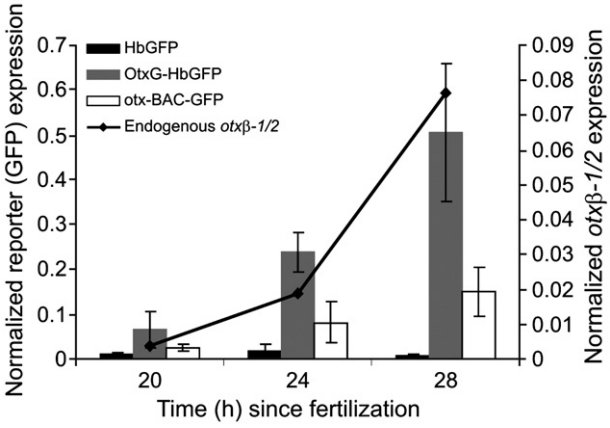

Fig. 4. Quantitative GFP reporter expression in embryos at 20 h, 24 h and 28 h after fertilization. GFP was driven by the DNA constructs HbGFP, OtxG-HbGFP or otx-BAC-GFP and expression in all cases was normalized to *ubiquitin*. Endogenous levels of *otxβ1/2* also normalized to *ubiquitin* expression are shown on the right ordinate. Values are based on three independent batches of 50–100 injected embryos. Error bars are ±1 standard deviation.

strict consensus sequence for Tbrain (A/G)GGTG(T/C)GA (Fig. 3, Supplemental Fig. 1A). Of the seven clusters tested for expression, only OtxG contained all of the predicted binding sites (including Tbrain) and also an Gata, Otx and Gata triplet (G4, O2, G5) within less than 100 bp.

OtxG drives correct reporter gene expression

OtxG-HbGFP was assayed for its ability to drive correct temporal (Fig. 4) and spatial expression (Fig. 5). Endogenous *otxβ1/2* expression first starts around 18 h and rises throughout development until at least 48 h after fertilization (Hinman et al., 2003b, and Fig. 4). GFP expression driven by OtxG-HbGFP follows a similar rise from 20 h to 28 h (Fig. 4). Quantitative measurements of transcript abundance also show that HbGFP alone drives only very low levels of GFP expression (Fig. 4). Quantitative abundance of GFP transcripts driven by the Otx BAC GFP recombinant was also determined and shown to have a similar profile (Fig. 4). Approximately 1/10th the number of BAC recombinant molecules as OtxG-HbGFP plasmid molecules was injected into zygotes for this assay. If GFP transcript abundance were normalized to the number of injected construct copies then BAC injected embryos would actually have a three to four fold higher level of reporter expression. Reporter expression profiles were similar, although overall lower, when OtxG was cloned into the expression construct EpGFPII (which uses the sea urchin endo16 basal promoter) or OpGFP (which uses the predicted basal promoter for *A. miniata otxβ1/2*) (data not shown).

The spatial expression of GFP transcription was also determined by WMISH at 26 h–30 h (Fig. 5A). At this stage of development, a thickening or slight invagination at the vegetal pole marks the endomesodermally fated tissue, while the majority of the remaining embryo is fated to ectoderm. The distinction between mesoderm and endoderm is difficult to determine this early in development. The reporter is expressed clonally (presumably due to the incorporation of the injected DNA construct during the first one to few rounds of cleavage) so 118 embryos from four separate injections were examined in order to achieve an accurate reflection of expression. 92% (108 embryos) showed some expression, where 89% (105 embryos) showing some expression in the ectoderm and 76% (90 embryos) in the endomesoderm. This mimics the expression of the endogenous *otxβ1/2* transcript at this time in development

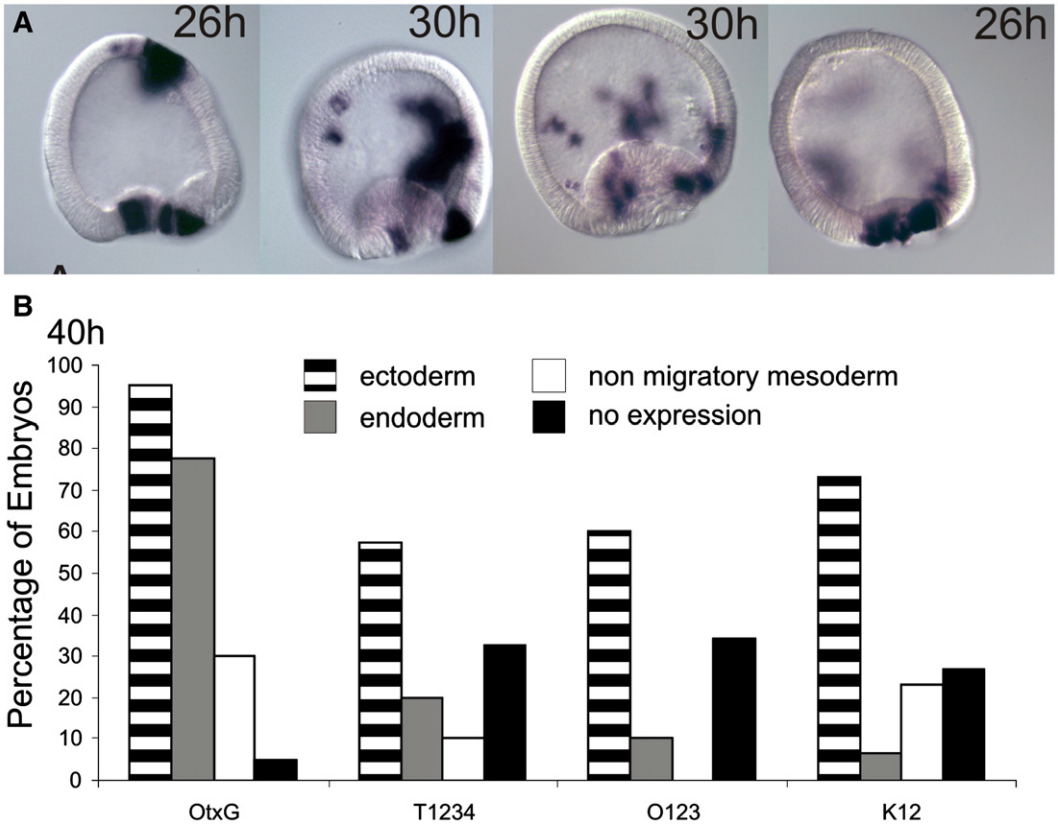

Fig. 5. GFP reporter expression driven by OtxG-HbGFP or specific mutations to this construct. (A) Examples of GFP expression driven by OtxG-HbGFP determined using whole mount in situ hybridization in 26 and 30 h embryos. (B) Percentage of embryos with GFP expression visualized using fluorescent microscopy, in ectoderm, endoderm, non-migratory mesoderm or with no visible expression at 40 h after fertilization. Constructs used to drive GFP expression are OtxG-HbGFP, or mutations to this construct, where T1234 mutation of all Tbrain consensus sites in OtxG, K12 mutation of all Krox/Blimp1 consensus sites and O123 mutation of all Otx consensus sites; see Supplemental Fig. 1 for details. GFP protein persists in the embryos at least until this time (40 h) so that the expression in any tissue may reflect the previous activation of GFP transcription in its progenitors. The visible expression of GFP can therefore be considered as the sum of all expression domains up to 40 h. Percentages sum to more than 100% as many embryos express in two or more tissue types. Data are based on three replicate experiments totaling approximately 150 embryos for each construct.

(Hinman et al., 2003b). The greater expression in the ectoderm perhaps reflects the greater proportion of the embryo fated to ectoderm at this stage.

Functional binding sites for all known regulatory inputs of otxβ1/2 are found within OtxG

The tissue specific regulation of the *otxβ1/2* transcript by Otx, Tbrain and Krox/Blimp1 was examined by disrupting all of the sites for each specific factor (i.e. O123, K12, T1234; see Supplemental Fig. 1) in OtxG and visually assaying GFP localization using fluorescence microscopy at 40 h (Fig. 5B). By this stage of development the embryo can be clearly divided into ectoderm, endoderm of the gut tube and migratory and non-migratory mesoderm (Hinman et al., 2003b). In embryos in which GFP was driven by the control OtxG-HbGFP construct, 95% of the embryos were found to exhibit some expression with all 95% having one or more patches of expression in the ectoderm, 77% with some expression in the endoderm and 30% in the non-migratory mesoderm (Fig. 5B). No expression was found in migratory mesenchyme. This mimics the expression of the endogenous *otxβ1/2* transcript which is expressed almost ubiquitously at the blastula stage but is restricted to the gut tube and ectoderm by 40 h (Hinman et al., 2003b).

When all of the potential Tbrain (T1234), Otx (O123) or Krox/Blimp1 (K12) sites were rendered nonfunctional, expression was dramatically reduced in the endoderm (Fig. 5B). Loss of Tbrain or Otx sites also dramatically reduced expression in the non-migratory mesoderm. The *krox/blimp1* product is restricted to the endoderm, while *otx* and *tbrain* are expressed throughout the endomesoderm (Hinman and Davidson, 2003; Hinman et al., 2003b).

The ability of these mutated forms of OtxG to drive reporter gene expression in the HbGFP construct was also quantitatively assayed during development at 20 h (early blastula), 24 h (late blastula) and 28 h (early gastrula) using QPCR (Fig. 6). When all four putative Tbrain sites were mutated (T1234) the levels of the GFP reporter transcripts were reduced to around 20% of that driven by the control OtxG construct from blastula (20 h) to early gastrula stage (28 h). Disruption of the three Otx sites (O123) similarly dramatically reduced GFP expression to less than half that of the control levels in earlier development, although the effect was less dramatic at 28 h. Conversely mutating core sequences in the Krox/Blimp1 sites (K12) did not have much effect at 20 h, but reduced reporter expression significantly by 24 h and 28 h. We also sought to examine the functionality of the combination of the two Gata sites surrounding one of the Otx sites, as it was shown in the sea urchin that this combination of factors is together necessary for correct expression of the *otxβ1/2* transcript (Yuh et al., 2004). When the two Gata sites most proximal to the transcription start and the Otx site between them were mutated (G45O2; see Fig. 3) there was a dramatic reduction in the GFP transcript abundance at 20 and 24 h to levels lower even than that found when all three otx sites (O123) were mutated (Fig. 6). Conversely, when the same Gata sites were mutated along with the most proximally located Otx site (G45O3), no significant effect in GFP abundance compared to the controls was found (Fig. 6).

Functional Tbrain is required for correct OtxG expression

The OtxG element also responds to the Tbrain input in *trans*. When embryos were co-injected with a morpholino antisense

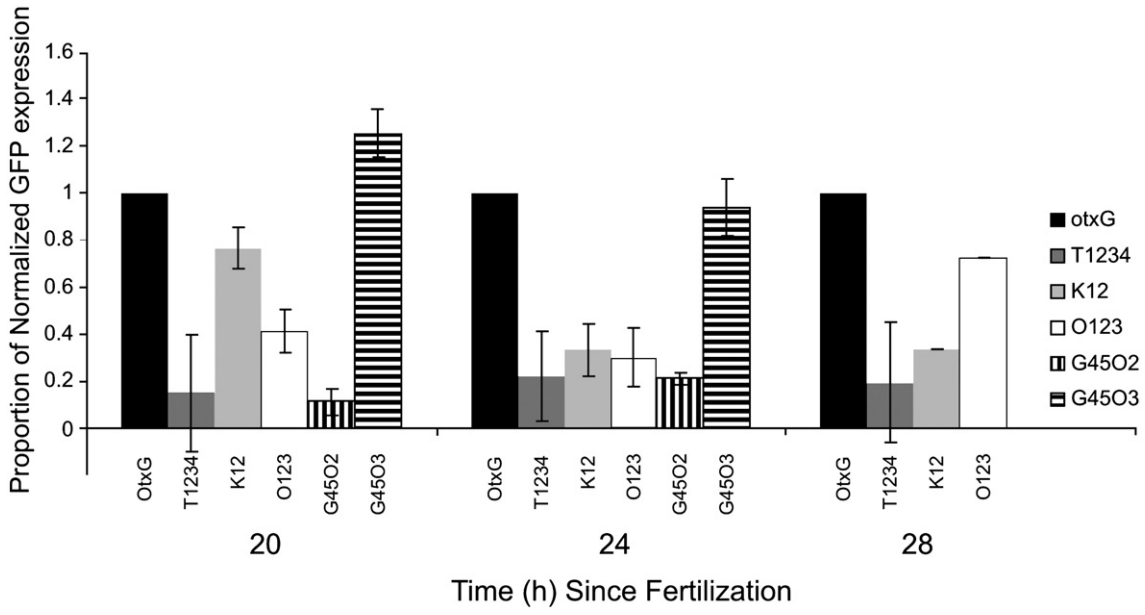

Fig. 6. Proportion of GFP transcript abundance relative to that driven by OtxG-HbGFP (OtxG) in embryos at 20 h, 24 h and 28 h determined using QPCR. Constructs are named as in Fig. 5, G45O2, mutation of Gata consensus sites number 4 and 5 (see Supplemental Fig. 1 and Fig. 3) and Otx site number 2 in OtxG-HbGFP; G45O3, mutation of Gata consensus sites number 4 and 5 (see Supplemantal Fig. 1 and Fig. 3) and Otx site number 3 in OtxG-HbGFP. Error bars are ±1 standard deviation based on 3 independent replicates of approximately 50 embryos for each construct.

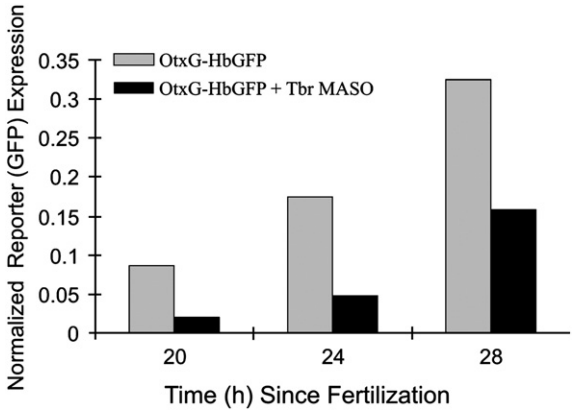

Fig. 7. Normalized expression of GFG driven by OtxG-HbGFP. Zygotes were injected either with either OtxG-HbGFP or with OtxG-HbGFP and a MASO against *Tbrain*. GFP expression was quantified at 20 h, 24 h and 28 h post fertilization. Data are based on two separate experiments of approximately 50 embryos each.

oligonucleotide against Tbrain and OtxG-HbGFP, GFP abundance was dramatically reduced throughout blastula and early gastrula (Fig. 7). This demonstrates that OtxG requires Tbrain protein in order to correctly direct expression.

Taken together, these results unequivocally demonstrate that OtxG is a *cis*-regulatory module regulating the endomesodermal expression of the sea star *otxβ1/2* gene and that it has functional binding sites for the four known GRN inputs, i.e. Gata, Krox/Blimp1, Otx and Tbrain. Thus, the inputs of the predicted GRN surrounding *otxβ1/2* are all demonstrated to occur at the direct *cis*-regulatory level. We cannot discount that other *cis*-regulatory modules also regulate embryonic *otxβ1/2* expression.

Cross-species expression analysis

Fig. 8 demonstrates that both of the sea star constructs OtxG-HbGFP and the Otx-BAC-GFP drive extensive GFP expression in the ectoderm and endoderm in the sea urchin embryo, while only less than 10% of embryos have any expression in the vegetal plate mesoderm and no expression was ever found in the skeletogenic/PMC lineage. This reflects endogenous *otxβ1/2* expression in the sea urchin. This experiment demonstrates an extraordinary conservation over 500 million years of evolution: The arrangement of binding sites within the sea star OtxG module and the structure of the proteins in the sea urchin are both sufficiently conserved to drive expression.

In the reverse experiment where SpOtx15/16pGL3 was injected into sea star embryos very little expression was observed compared to controls (Fig. 9).

There are two possibilities for this failure for SpOtx15/16pGL3 to express in the sea star. Firstly, that the sea star proteins have reduced affinity for the sea urchin binding sites (although the reverse is shown to not be true) and secondly that

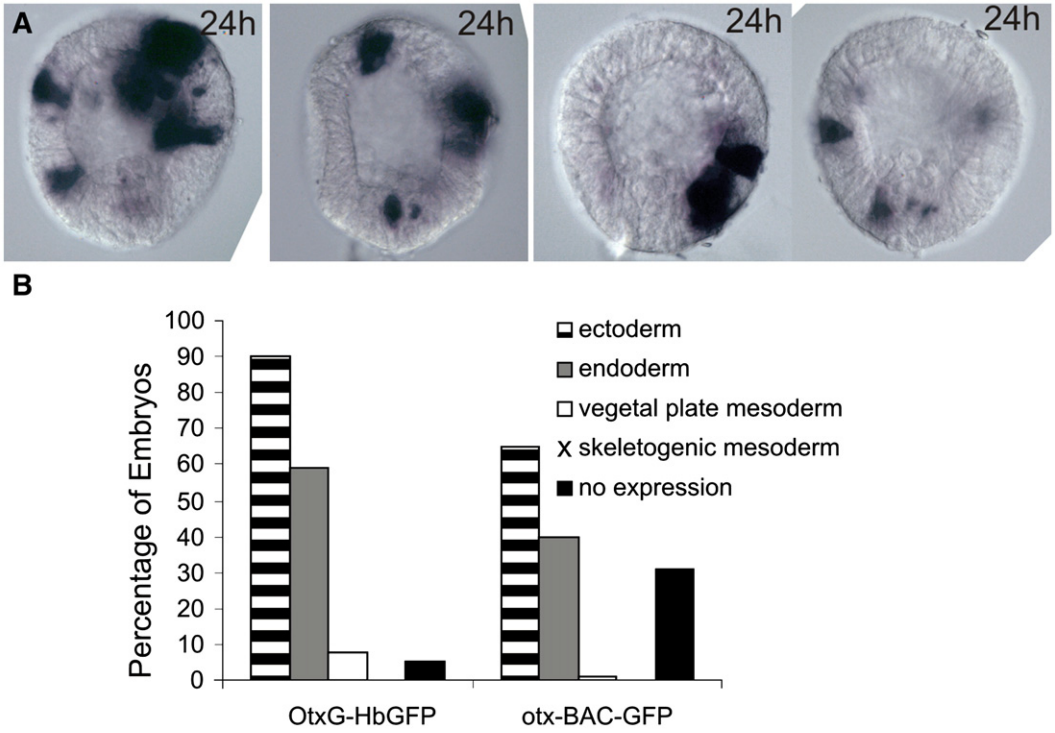

Fig. 8. Spatial expression of GFP reporter assayed in sea urchin embryos driven by sea star-specific DNA constructs. (A) Examples of GFP expression driven by OtxG-HbGFP, determined using WMISH in 24 h sea urchin embryos. (B) Percentage of sea urchin embryos with GFP expression (visualized by fluorescent microscopy) in ectoderm, endoderm, vegetal plate mesoderm, skeletogenic mesoderm or with no visible expression 30 h after fertilization. At this stage of development the various cell types which will emerge from the vegetal plate are not distinguishable so we make no attempt to characterize expression further for this lineage. Constructs used to drive GFP expression are the starfish OtxG-HbGFP or the starfish otx-BAC-GFP. GFP protein persists in the embryos at least until this time so that the expression in any tissue may reflect the previous activation of GFP transcription in its progenitors. Percentages sum to more than 100% as many embryos express in two or more tissue types. Results are based on two separate injections of approximately 50 embryos each.

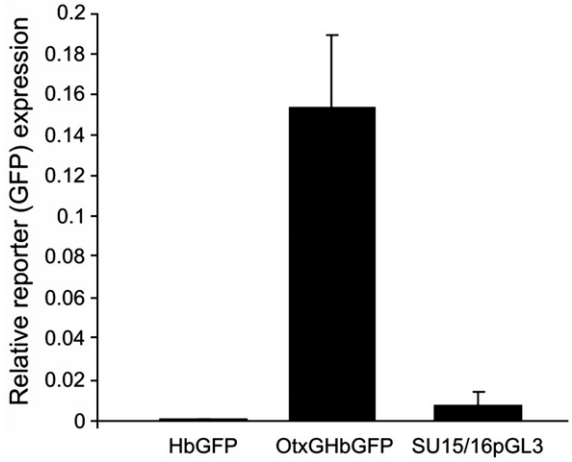

Fig. 9. Quantitative GFP transcript abundance in sea star embryos driven by the expression vector only (HbGFP), the sea star OtxG-HbGFP construct or the sea urchin Otx15/16pGL3 construct. Values were determined using QPCR and are normalized to *AmUbiquitin* and are based on three replicate experiments with approximately 50 embryos each. Error bars are ±1 standard deviation.

binding sites are missing or arranged incorrectly in the sea urchin *cis*-regulatory DNA and cannot bind the proteins appropriate for correct expression in the sea star.

We performed a rescue experiment in order to distinguish between these alternative hypotheses. Reporter expression increased around five fold when a small region of the sea star OtxG was inserted into the sea urchin Otx15 *cis*-regulatory module (Fig. 10 and Supplemental Fig. 2). This inserted region contained three binding sites for Tbrain, including the strict consensus site (T3), and also one each for Otx and Gata (Fig. 10B). We sought to determine the portion of this increase that could be attributed to the presence of Tbrain sites in this region by generating another construct with the same insert except that the three Tbrain sites were mutated to prevent binding. This mutated Tbrain form of the construct reduced GFP expression by about 50% although it was not reduced to the level driven by the original sea urchin Otx15/16pGL3 construct. This shows that the failure for SpOtx15/16pGL3 to express can at least in part be explained by the absence of Tbrain binding sites.

Discussion

We identify and characterize here the *cis*-regulatory module controlling *otxβ1/2* expression in the sea star *A. miniata*. Through mutational analyses we show that each of the four inputs predicted by *trans* perturbation analyses (i.e. Gatae, Krox/Blimp1, Tbrain and Otx) have functional binding sites within the identified element. The functionally equivalent *cis*-regulatory module has also been characterized in the sea urchin *S. purpuratus*, where it was shown that only three of these same factors, Gatae, Krox/Blimp1 and Otx, also bind to regulate expression. *S. purpuratus tbrain* is not co-expressed with *otxβ1/2* and functions instead to produce the embryonic skeleton which is considered an evolutionary novelty of the sea urchins (Willmer, 1994). As *tbrain* orthologues are broadly expressed within the endomesoderm in a variety of other deuterostomes this evolutionary change to a skeletogenic function is a derived feature of sea urchins. The acquired role of *tbrain* in the skeletogenic GRN of sea urchins is a perfect example of an evolutionary co-option in transcription factor function. Accompanying this co-option is a loss of functional Tbrain binding sites within a direct endomesodermal regulatory target. *Trans*-species expression of the constructs confirms that loss of Tbrain sites within the *cis*-regulatory module of *otxβ1/2* destroys the functionality of the module when it is reinserted into the pleisiomorphic regulatory environment. On the other hand there is a striking conservation in the distribution and additive function of a Gata/Otx/Gata binding site triplet within the cis-regulatory modules in both taxa. The requirement for synergism between the Gata and Otx factors on the *cis*-regulatory module in both echinoderms may explain the conserved distribution of this triplet of sites and provides a simple functional explanation for maintenance of this node of the GRN kernel.

Cis-regulatory module structure in the sea star

GRN analyses demonstrated that four regulatory factors are required for the correct expression of *otxβ1/2* in *A. miniata*; *viz* Krox/Blimp1, Gatae, Tbrain and Otx (Hinman et al., 2003a). One of the aims of this study is to determine if these inputs are

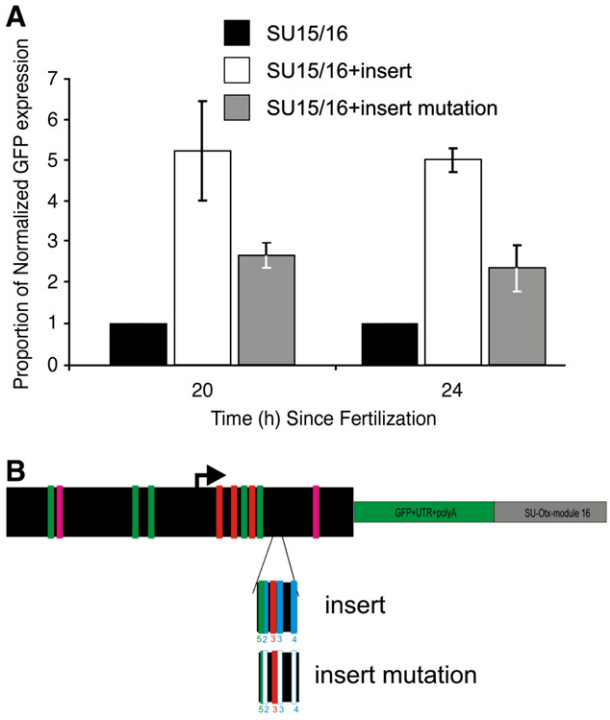

Fig. 10. (A) Proportion of GFP expression driven by two modified sea urchin constructs compared with the unmodified sea urchin module Otx15/16pGL3 construct, in starfish embryos at 20 h and 24 h after fertilization. (B) The endogenous sea urchin construct containing modules 15/16 was modified by adding a region of the starfish module OtxG containing single binding sites for Gata (G5), Otx (O3) and three binding sites for Tbrain (T2, 3, 4), this construct is named "SU15/16+ insert" (see details in Supplemental Fig. 2). An alternative construct was also generated in which the three inserted Tbrain sites were mutated (this is named "SU15/16+ insert mutation") as indicated in Supplemental Fig. 1.

direct. We searched for clusters of the known consensus binding sites for Otx, Gata and Krox/Blimp1 in 100 kb of sequence surrounding the *otx* gene. Of the eight identified clusters only one, OtxG, drove any reporter expression, and both the spatial and temporal profile mirrored that of the endogenous *otxβ1/2* transcript (Figs. 4 and 5). The expression profile driven by OtxG is not consistent with that of either of the other transcript forms of *otx* (see Hinman et al., 2003b). We are thus confident that OtxG is a regulatory module normally used to drive *otxβ1/2* expression from at least blastula to early gastrula stage in *A. miniata*.

Comparisons of the nucleotide sequence of OtxG and that of the *cis*-regulatory module regulating *otxβ1/2* expression in the sea urchin, SpOtx15, showed no significant contiguous patches of sequence conservation when using Blast or Family Relations (Brown et al., 2005) alignment algorithms. Cameron et al. (2005) found high rates of single nucleotide substitutions and small insertion/deletions occurring within species of echinoids that diverged within the last 50 million years. Therefore, it would be expected that random mutation alone should have sufficiently obliterated any non-selected sequence identity during the 500 million years (Wada and Satoh, 1994) separating *A. miniata* and *S. purpuratus*. Despite this enormous period of independent evolution, however, we did find a striking level of conservation of the numbers and arrangements of the binding sites distributed within the modules (Fig. 3). Both OtxG and SpOtx15 contain five sites for Gata, three for Otx and two for Krox/Blimp1. Another common feature is the presence of an Otx site (O2 or O3) closely flanked by two Gata sites (G45). The conservation of numbers of sites is intriguing as studies in both organisms have shown that not all are functional, at least in the laboratory assays we could apply. We also identified four consensus Tbrain sites within OtxG. The OtxG module is located approximately 11 kb downstream of the last *otx* exon, in contrast to SpOtx15 which is located immediately upstream and in the 5′UTR of the *otxβ1/2* transcript. Since there is no surrounding sequence identity we are unable to determine if one or both of these modules have been translocated in relative location from that in their common ancestor or have arisen *de novo* in a new location through the random accumulation of appropriate binding sites.

Site directed mutagenesis confirmed that the binding sites determined on the basis of consensus sequence are functional in the sea star (Figs. 5 and 6). This analysis also provided a more detailed understanding of the logic processing underlying the control of *Amotxβ1/2* expression. When all instances of each binding site for Otx, Tbrain or Krox/Blimp1 are mutated so that the core binding motif is disrupted, there is a dramatic reduction of GFP expression in the endoderm (Figs. 5 and 6). This confirms that each of these three inputs is necessary, i.e. unless all three factors are bound to OtxG, expression is severely depressed.

We also show that if the combination of the two Gata sites and the Otx site they flank are mutated (G45O2), expression is reduced further than when only all Otx sites or the same two Gata sites and an alternatively positioned Otx site are perturbed (Fig. 6). This indicates that synergism between these Gata and Otx sites is obligatory, i.e. that the *cis*-regulatory control region for these proteins operates as an AND logic processor. This is exactly as the *cis*-regulatory control region for these proteins is proposed to function in the sea urchin (Yuh et al., 2004). Mechanistically, it is likely that, in both these echinoderms, the Otx and Gatae proteins must interact to form a stable transcriptional activation complex and hence the proximity and ordering of these binding sites in the *cis*-regulatory module have been maintained throughout immense periods of evolution. This simple functional constraint may explain the preservation of this part of the GRN (i.e. that Gatae and Otx have been maintained as inputs into the *otxβ1/2* transcript) which in turn explains the conservation of this regulatory kernel.

Conservation and change of the cis-regulatory logic controlling the A. miniata and S. purpuratus otxβ1/2 transcripts

Fig. 11 provides a comparison of the GRN architectures surrounding *otxβ1/2* in *A. miniata* and *S. purpuratus*. In *S. purpuratus*, the early form of Otx, Otxα, initially activates *krox/blimp1*, which in turn provides the first known input into *Spotxβ1/2* (Yuh et al., 2004). The input from AmKrox/Blimp1, however, is not required for the initial activation of the OtxG regulatory module because loss of functional binding does not impact early reporter expression, although it is required in later development (Fig. 6). Also the maternal form of *otx* in sea stars is the *otxβ3* transcript which is not expressed within the endomesoderm and hence cannot have a role in Krox/Blimp1 regulation. It seems likely that Krox/Blimp1 provides a spatial input into *otxβ1/2* (just as it also does in *S. purpuratus*) since loss of these sites most significantly reduces reporter expression in the endoderm while ectoderm and mesoderm territories are hardly affected (Fig. 5). Krox/Blimp1 expression is spatially restricted to the endoderm (Hinman and Davidson, 2003). Thus, in the sea star, some other factor must provide the early input into *otxβ1/2* and initiate the *otx*/*krox*/*gatae* feedback loop. This function we suggest is provided, at least in part, by Tbrain.

Tbrain is an ideal candidate because it is expressed earlier than *otxβ1/2* (Fig. 1) but is similarly spatially restricted. Notably, when all of the Tbrain or Otx sites are disrupted, reporter expression is dramatically reduced from early development (Fig. 6). While *otxβ1/2* obviously cannot function as an autoactivator it may have a stabilizing, ‘lockdown’ function on its own expression, just as it does in *S. purpuratus* (Yuh et al., 2004). None of the other forms of Otx provides the early input into *otxβ1/2* as they are expressed inappropriately. The *Amotxβ3* transcript is expressed maternally and throughout development but is restricted to the ectoderm and therefore cannot account for *otxβ1/2* activation in the endomesoderm. The other form, *otxα*, is restricted to the early endomesoderm but is first detected at the same time as *otxβ1/2* (Hinman et al., 2003b).

Evolutionary changes underlying a dramatic reorganization of GRN architecture

The data presented here, combined with previous knowledge of the sea urchin and sea star endomesoderm GRN (Davidson

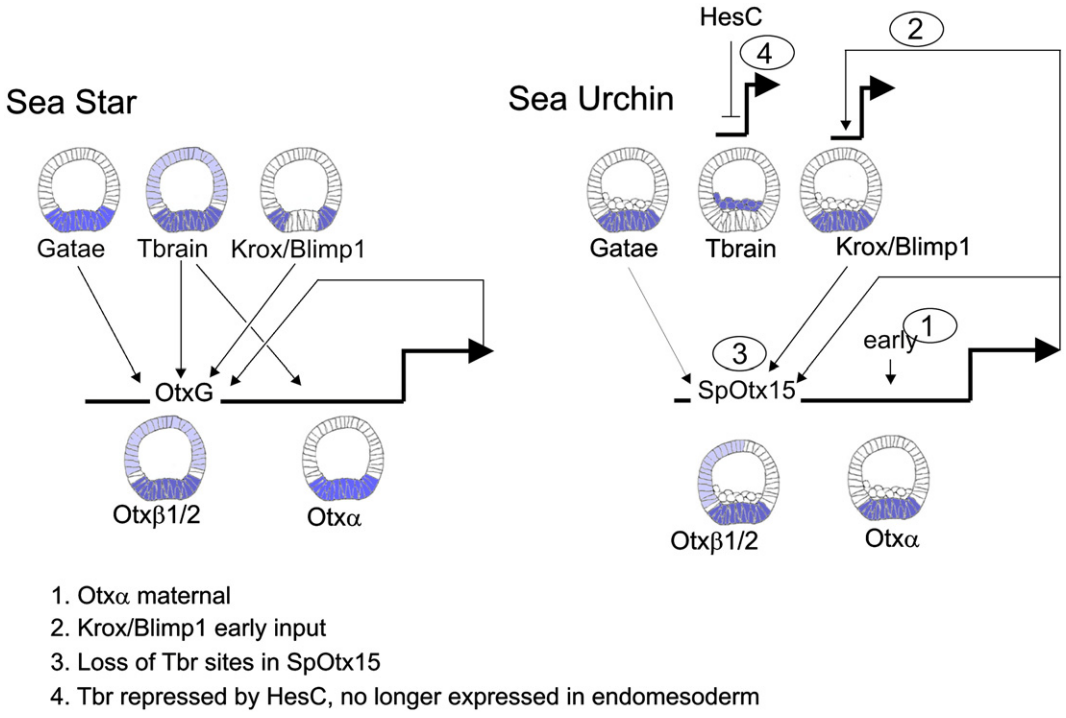

Fig. 11. Schematic representation of GRN architecture surrounding the *otx* gene in sea urchin and sea star. Bold lines with arrows represent *cis*-regulatory module and arrowheads into these represent regulation from the named factor. Embryo schematics are colored according to spatial expression of the below named factors determined by WMISH.

et al., 2002a,b; Davidson, 2006; Hinman et al., 2003a; Fig. 11), suggest that there were a series of evolutionary events leading to the co-option of Tbrain expression and function in the sea urchin (see notes in Fig. 11). Maternal expression of the alternatively spliced form of otx, *otxα*, may have been one of them. This factor could now serve as an early driver of *Krox/Blimp1* expression throughout the endomesoderm (Livi and Davidson, 2006; Smith et al., in press). The Krox/Blimp1 protein could now have bound the ancestrally existing target sites of the *otxb1/2 cis*-regulatory module earlier in development. In this way Krox/Blimp1 and Tbrain might have become at least partially redundant as both proteins activated early *otxβ1/2* expression. Thus evolutionary loss of *tbrain* expression in the endomesoderm could have occurred while correct function of *otxβ1/2* was maintained. Without the need for *tbrain* expression in the endomesoderm, functional binding sites within the *S. purpuratus otxβ1/2 cis*-regulatory module would have accumulated disruptive mutations and have been lost. We know that *tbrain* is repressed everywhere outside of the skeletogenic lineage in *S. purpuratus* by the transcription factor HesC (Revilla-i-Domingo et al., 2007), another evolutionary event that accounts causally for the absence of its expression throughout the endomesoderm (see Fig. 11).

Cross-species expression confirms evolutionary change at cis-regulatory sequence level

Our inter-specific functional experiments test some inferences from the above. One prediction is that the sea star *otxβ1/2 cis*-regulatory module OtxG should have all of the binding sites needed to correctly integrate the upstream regulatory state of the sea urchin and thus drive reporter expression appropriately (see Fig. 11). And indeed, when injected into *S. purpuratus* eggs, the sea star OtxG *cis*-regulatory module is able to drive very intense GFP expression with a similar spatial restriction as displayed by the endogenous sea urchin *otxβ1/2* (Fig. 8). This demonstrates also that the sea urchin proteins are conserved sufficiently that they can bind effectively to the DNA sequences present in *A. miniata* and that the arrangement, numbers and types of binding sites within the module are well enough conserved to regulate expression. It should be noted that, although the sea star OtxG module does not use an early Krox/Blimp1 input to initiate expression in the sea star, these sites are present and needed for later expression. Thus OtxG can correctly integrate this input in the sea urchin regulatory environment, where Krox/Blimp1 is present early.

The postulated evolutionary exchange in function between Tbrain and Krox/Blimp1 suggests on the other hand that the SpOtx15 module will not be able to respond to the proteins present in the sea star blastula to drive appropriate reporter expression. The sea urchin regulatory module SpOtx15 does not possess the Tbrain sites needed to drive *Amotxβ1/2*, and Krox/Blimp1 is not expressed appropriately in the sea star to activate *otxβ1/2* in the absence of functional Tbrain sites. This prediction is confirmed. When the SpOtx15 reporter construct is injected into *A. miniata*, almost no expression is detected (Figs. 9 and 10). This interpretation is further supported by the demonstration that the addition of Tbrain sites into the SpOtx15 module can increase reporter expression (Fig. 10). An alternative rescue experiment would be to introduce Krox/Blimp1 protein into the

endomesoderm of early blastula stage sea star embryos. We predict that this should also drive SpOtx15 reporter expression. However, over-expressing transcription factors that have multiple targets in the zygote is too blunt an experimental axe and so this experiment was not attempted.

Conclusion

This work shows how evolution of a *cis*-regulatory module accommodates a dramatic reorganization in GRN architecture. The *otxβ1/2* gene has an essential and conserved role in the specification of the endomesoderm in the distantly related echinoderms, the sea star *A. miniata* and the sea urchin *S. purpuratus*. Its expression and function are conserved despite alterations in upstream GRN architectures, i.e. a loss of regulation by Tbrain in the sea urchin. We postulate that the Krox/Blimp1 factor takes over the role of Tbrain in the sea urchin and we find a corresponding loss of Tbrain sites within the sea urchin *otxβ1/2 cis*-regulatory module. Conversely, we show that highly conserved regulatory inputs into *otxβ1/2* from Otx and Gatae have been maintained during this same period of evolution. This high conservation may be the consequence of the need for the synergism of both factors to bind the *cis*-regulatory module in both taxa and may provide a simple functional explanation for the conservation of the "*otx* node" of the GRN kernel.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.09.006.

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